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USP33 mediates Slit-Robo signaling in inhibiting colorectal cancer cell migration

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Originally discovered in neuronal guidance, the Slit-Robo pathway is emerging as an important player in human cancers. However, its involvement and mechanism in colorectal cancer (CRC) remains to be elucidated. Here, we report that Slit2 expression is reduced in CRC tissues compared with adjacent noncancerous tissues. Extensive promoter hypermethylation of the Slit2 gene has been observed in CRC cells, which provides a mechanistic explanation for the Slit2 downregulation in CRC. Functional studies showed that Slit2 inhibits CRC cell migration in a Robo-dependent manner. Robo-interacting ubiquitin-specific protease 33 (USP33) is required for the inhibitory function of Slit2 on CRC cell migration by deubiquitinating and stabilizing Robo1. USP33 expression is downregulated in CRC samples, and reduced USP33 mRNA levels are correlated with increased tumor grade, lymph node metastasis and poor patient survival. Taken together, our data reveal USP33 as a previously unknown tumor-suppressing gene for CRC by mediating the inhibitory function of Slit-Robo signaling on CRC cell migration. Our work suggests the potential value of USP33 as an independent prognostic marker of CRC.

Colorectal cancer (CRC) is the third most common cancer and the second most common cause of cancer death worldwide.[1] The incidence of CRC is increasing rapidly in China and other Asian countries. Survival rate of CRC varies with the disease stage at diagnosis, from ~90% for localized cancer to ~10% for distant metastatic cancer. Despite recent advances in diagnostics and therapy, CRC remains a major health problem worldwide. One of the major obstacles to effective treatment of CRC is the high metastasis and recurrence rates as a result of the intrinsic feature of high migration and invasion ability of CRC cells. Understanding the molecular mechanisms of CRC metastasis is critical for developing new antimetastasis strategies.

Slits (Slit1-3) are secreted, extracellular matrix-associated glycoproteins and ligands for the neuronal guidance receptor Roundabout family (Robo1-4). The Slit-Robo pathway is crucial for neuronal guidance and cell migration.[2–5] Recent data show that this pathway also plays important roles in many physiological and pathological processes outside of the nervous system, especially in human tumorigenesis.[6,7] Slit2 expression is frequently downregulated in a variety of cancer types, including CRC.[8] However, the molecular mechanisms underlying Slit-Robo signaling in human cancers are not clear.

Although efforts have been made to elucidate the role of Slit-Robo signaling in CRC, the results are contradictory.[8–10] For example, Dallol *et al.*[8] reported that Slit2 is frequently inactivated in CRC and suppresses the growth of CRC cells, whereas Zhou *et al.*[9] showed that the N-terminal domain of Slit2 could induce malignant transformation of colorectal epithelial cell and tumor metastasis. Interestingly, a recent paper revealed that Slit-Robo could inhibit CRC cell migration.[10] Therefore, further study is necessary to elucidate the precise role and underlying mechanisms of Slit-Robo signaling in CRC.

Key words: Slit2, Robo1, USP33, cell migration, colorectal cancer

Abbreviations: BSP: bisulfite-sequencing PCR; CM: conditioned medium; CRC: colorectal cancer; IP: immunoprecipitation; MSP: methylation-specific polymerase chain reaction; NCT: noncancerous tissues; qRT-PCR: quantitative reverse transcription polymerase chain reaction; qPCR: quantitative polymerase chain reaction; siRNA: small interfering RNA; USP33: ubiquitin-specific protease 33

Additional Supporting Information may be found in the online version of this article.

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What's new?

The Slit-Robo pathway functions in axon guidance and cell migration and influences various pathological processes outside the nervous system, including tumorigenesis. Here, the Robo ligand and putative tumor suppressor Slit2 was found to serve as an inhibitor of cell migration in colorectal cancer (CRC). Its inhibitory activity was dependent on Robo1 and was enhanced by overexpression of USP33. USP33 mediated Slit2 activity via Robo1 deubiquitination and stabilization. In addition, Slit2 and USP33 expression was reduced in human CRC tissue. The data suggest that USP33 is a tumor suppressor in CRC and that its expression is predictive of survival.

Ubiquitin-specific protease 33 (USP33), a newly identified Robo1-interacting molecule, is required for midline crossing and the responsiveness of commissural axons to Slit and for Slit-Robo signaling in inhibiting breast cancer cell migration.[11,12] These data suggest an important role of USP33 in Slit signaling. In this study, we present evidence that Slit2 is downregulated in CRC and inhibits CRC cell migration in a Robo-dependent manner. USP33 mediates the inhibitory function of Slit2 on CRC cell migration by deubiquitinating and stabilizing Robo1. Interestingly, in CRC cells, Slit2 treatment also results in an increase in Robo1 on the cell surface. Consistently, USP33 expression is downregulated in CRC samples and appears to be an independent prognostic factor for CRC.

Material and Methods

Human tissues and cell lines

Seventy-nine de-identified human primary CRC tissues and their adjacent noncancerous tissues (NCTs) were collected between 2004 and 2008 at the Affiliated Hospital of Jiangnan University and Fudan University Shanghai Cancer Center. The detailed information of patients is included in Supporting Information Table S1. All of the samples were obtained following national guidelines with informed consent and with approvals from the Clinical Research Ethics Committees of the Affiliated Hospital of Jiangnan University and Fudan University Shanghai Cancer Center.

HEK-293 cell line and six human CRC cell lines, including HCT8, HCT116, LoVo, Caco2, DLD1 and HT29, were purchased from American Type Culture Collection and cultured in media (Hyclone) supplemented with 10% fetal bovine serum (Gibco).

Antibodies, plasmids, small interfering RNA and stable cell lines

Mouse anti-Flag (M2), anti-beta-actin and anti-beta-tubulin were from Sigma-Aldrich; mouse anti-HA (16B12), anti-GFP and anti-myc (9E10) were from Covance Laboratories; anti-Ub was from Santa Cruz Biotechnology; anti-Robo1 was from Abcam; anti-USP33 was from Bethyl Laboratories. The Robo1-HA, Robo1-myc, RoboN (the soluble extracellular domain of Robo1), GFP-USP33 and Flag-tagged ubiquitin (Flag-UB) plasmid constructs were generated as described.[5,12–14] Slit2 and GFP-USP33 were subcloned into the lentivirus plasmid pLVX-IRES-Neo (Clontech). Duplex small interfering RNAs (siRNAs) were purchased from GenePharma (Shanghai, China).[12] Plasmids and siRNA transfection were performed using Lipofectamine 2000 (Invitrogen). HEK293 cells stably expressing Robo1-HA, RoboN or Slit2-myc were constructed as previously described.[5] The detailed information of conditioned medium (CM) was described in Supporting Information Materials and Methods. LoVo cells stably expressing Slit2, GFP-USP33 or Slit2/GFP-USP33 were constructed using lentivirus transduction and G418 selection.

RNA extraction and quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from cells or tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized using PrimeScript RT reagent kit (TaKaRa, Japan), and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were conducted using SYBR Premix Ex Taq (TaKaRa) with beta-actin as an internal control. Both qRT-PCR and radioactive PCR as previously described[11] were used to detect different gene expression in the CRC cell lines. The primers used for PCR analysis are listed in the Supporting Information Data (Supporting Information Table S2).

Promoter methylation analysis

Genomic DNA was isolated from CRC cell lines, CRC tissues and adjacent NCTs using General Allgene kit (CWbio, China). Genomic DNA samples were bisulfite-modified as previously described.[15] The bisulfite-treated DNA samples were amplified using bisulfite-sequencing PCR (BSP) primers located in the promoter region of human Slit2 gene. The purified BSP products were directly sequenced with the methylation status of each CpG site determined as previously described.[8] To perform methylation-specific PCR (MSP) analysis, the bisulfite-treated DNA was subjected to PCR, and the PCR products were analyzed using 2% agarose gel electrophoresis. The primers for the BSP and MSP assays were designed using MethPrimer (http://www.urogene.org/methprimer/; Supporting Information Table S2).

Cell viability and cell migration assays

Cell viability analysis was performed using the MTT assay. A modified wound-healing assay for cell migration was performed as we have described previously,[11] and the detailed

information was presented in the Supporting Information Materials and Methods. To mimic the *in vivo* migration and invasion behavior of tumor cells, a three-dimensional coculture assay was performed as previously described.[13,16] In addition, cell migration assay was performed using the Transwell method as previously described.[17] For the *in vivo* metastasis assays, 2×10^6 LoVo cells stably expressing Slit2, USP33 or the control vector were suspended in 100 μL of PBS and were injected into the caudal vein of each nude mouse. The mice were sacrificed after a period of 5–6 weeks and examined for the lung metastasis of tumors.

Western blotting, immunoprecipitation and immunohistochemical staining

Western blotting and immunoprecipitation (IP) analyses were performed as previously described.[11,12] To detect the expression levels of the USP33 and Robo1 protein, immunohistochemical staining was performed on 4 μm sections of paraffin-embedded CRC tissues as previously described.[17]

Deubiquitination assay

HCT116 cells were transiently transfected with plasmids encoding Flag-UB, Robo1-myc and GFP-USP33 or siUSP33. After 48 hr, cells were treated with 10 μM MG132 (Peptides International) for 6 hr and then lysed using IP buffer (1% Triton-100, 10% glycerol and 1 mM EDTA in TBS buffer). The cell lysates were incubated with anti-Flag to precipitate Flag-tagged ubiquitinated proteins for 3 hr. The cell lysates were then incubated with protein A/G agarose beads for 3 hr. Following washes, the beads were eluted in SDS loading buffer, separated by SDS-PAGE and detected using Western blotting.

Cell surface biotinylation assay

Robo1-HA-expressing HCT116 cells were plated in 10-cm dishes and transfected with USP33-specific siRNA (siUSP33) or a control siRNA (siCtrl). Forty-eight hours after transfection, the cells were treated with recombinant Slit2 for 10 min. Then, cell surface proteins were biotinylated and isolated using Pierce Cell Surface Protein Isolation Kit (Thermo Scientific) according to the published protocol.[11] Biotinylated cell surface proteins were examined by immunoblotting using anti-HA.

Statistical analysis

The results are presented as the mean values ± SEM. The data were subjected to Student's *t*-test, Mann–Whitney *U* or Kruskall–Wallis test. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was applied for comparisons. The Cox proportional hazards regression model was used to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs), representing the overall relative risk of death that was associated with different variables. A p value of less than 0.05 was considered statistically significant. The Graphpad prism 5.0 software (GraphPad Software) and SPSS 16.0 package (SPSS) were used for statistical analyses and scientific graphing, respectively.

Results

Slit2 expression was downregulated in CRC

The mRNA levels of known genes in Slit-Robo signaling were examined in six CRC cell lines using radioactive PCR. The Slit2 gene expression was detected in HT29 cells, but only at low levels in LoVo, HCT116 and Caco2 cells, and not detectable in the other two cell lines. Robo1 expression was detected in HT29 and LoVo cells (Supporting Information Fig. S1*a*). Based on the Slit2 and Robo1 expression levels and the *in vitro* migration ability of these CRC cell lines, we focused on LoVo and HCT116 for the subsequent analysis.

To assess the expression of Slit2 and Robo1 in CRC samples, we first analyzed their expression in a published CRC microarray data set (Supporting Information Table S3).[18] Compared to NCTs ($n = 43$), the Slit2 mRNA levels were significantly downregulated in CRC tissues ($n = 180$) ($p < 0.0001$, Fig. 1*a*), whereas no significant difference was found in the Robo1 mRNA levels between CRC and NCT samples. We then used qRT-PCR to validate the downregulation of Slit2 mRNA in an independent CRC cohort we collected. Our data revealed that Slit2 expression was downregulated by more than 1.5-fold in 55.7% (44/79) of CRC tumors compared with their corresponding NCTs ($p < 0.0001$, Fig. 1*b*). No significant association was found between the Slit2 expression and patient age, gender, tumor stage, tumor size, or survival.

The promoter of the Slit2 gene was hypermethylated in CRC

To investigate the mechanisms underlying the suppression of Slit2 expression in CRC, we first analyzed datasets obtained from an online database (http://www.cbioportal.org/public-portal/). Genetic mutations in the human Slit2 gene was detected in approximately 4%–7% of tumors among different CRC cohorts, suggesting that other mechanisms may result in Slit2 inactivation in CRC (Supporting Information Fig. S2). Promoter hypermethylation is an important mechanism that results in gene silencing and has been reported to mediate the Slit2 inactivating.[8,19] A negative relationship was observed between the Slit2 mRNA and promoter methylation levels (Pearson $r = -0.346$, $p < 0.0001$, Fig. 1*c*), suggesting a role of DNA hypermethylation in silencing Slit2 expression in CRC. We then examined the promoter methylation status of Slit2 in CRC cell lines and clinical samples. The Slit2 promoter was found to be methylated in five of the six cell lines based on the BSP results (Fig. 1*d*). CRC cell lines with a hypermethylated Slit2 promoter showed no detectable Slit2 mRNA expression (DLD1, HCT8), and CRC cells with a partially methylated Slit2 promoter showed reduced Slit2 mRNA expression (HCT116, Caco2 and LoVo), whereas HT29 cells with an unmethylated Slit2 promoter showed the highest Slit2 expression among the six cell lines (Fig. 1*d* and

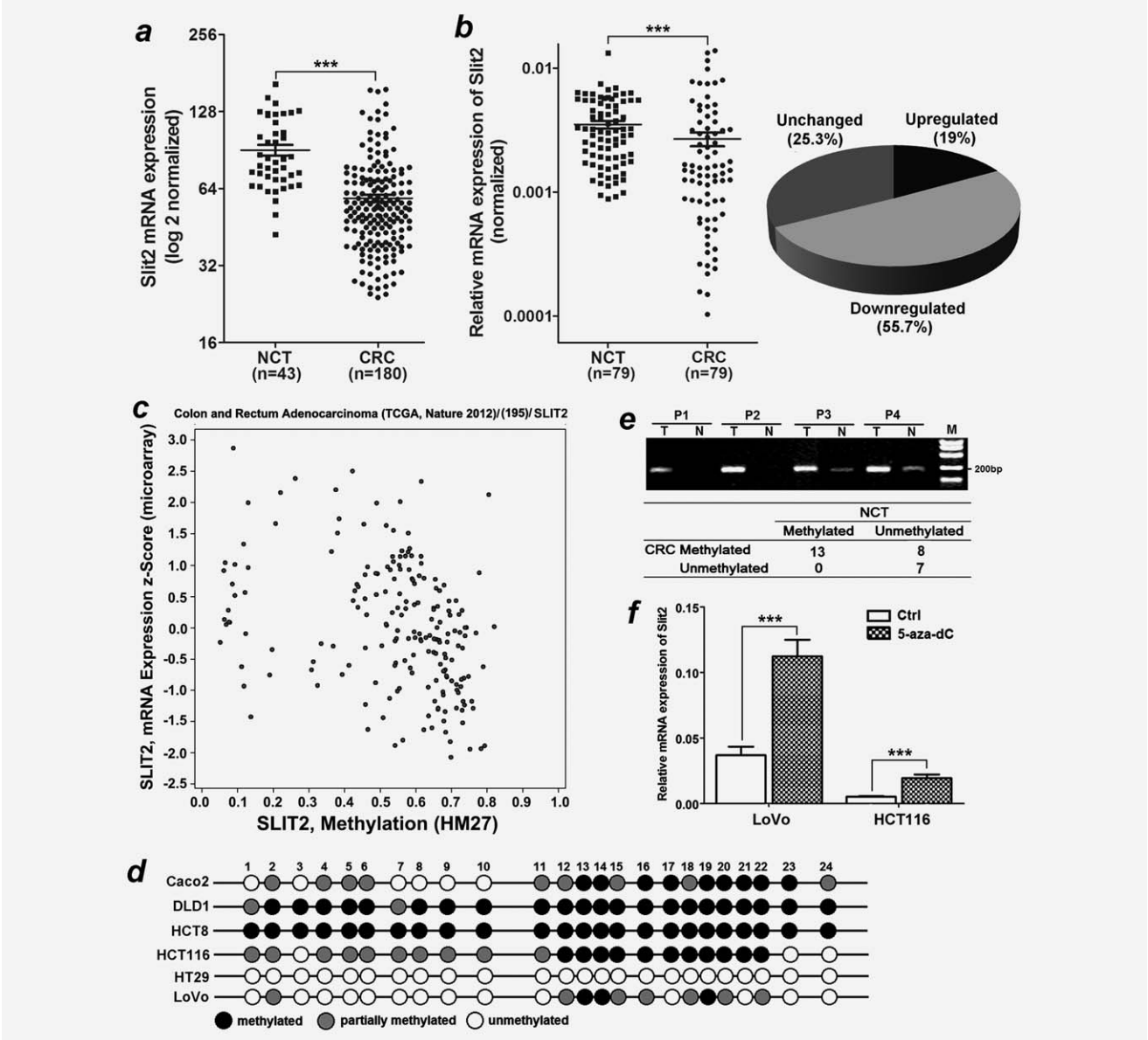

Figure 1. Promoter hypermethylation contributes to Slit2 downregulation in CRC. (*a*) and (*b*) Slit2 mRNA expression was downregulated in CRC tissues from two different cohorts. The data were obtained from a published microarray dataset (*a*)[18] and an independent test corhort (*b*). Slit2 mRNA levels were detected in 79 paired CRC and adjacent noncancerous tissues (NCTs) using quantitative real-time PCR (*b*). Slit2 mRNA expression was reduced by more than 1.5-fold in 55.7% (44/79) of CRC tumors compared with their corresponding NCTs. Mann–Whitney *U* test; $***p < 0.001$. (*c*) Slit2 mRNA levels were negatively correlated with the methylation levels of the Slit2 promoter (Pearson $r = -0.346$, $p < 0.0001$). The data were obtained from the cBioPortal for Cancer Genomics (http://cbio.mskcc.org/su2c-portal/). (*d*) DNA methylation analysis of Slit2 in 6 CRC cell lines using a bisulfite-sequencing PCR assay. Circles mark different CpG cites in the analyzed sequence. (*e*) DNA methylation analysis of Slit2 in CRC tissues using methylation-specific PCR assay. The methylation ratio of Slit2 in CRC tissues (75.0%) was significantly higher than that in NCTs (46.4%; $p = 0.029$). (*e*) CRC tumors with hypermethylated Slit2 promoter showed decreased Slit2 expression compared with those with hypomethylated Slit2. P1-P4, different patients; N, adjacent noncancerous tissue; T, tumor tissue; M, DNA marker. (*f*) Quantitative analysis of Slit2 mRNA expression in CRC cells treated with DNA methyltransferase inhibitor (5-aza-dC). Following treatment of HCT116 and LoVo cells with 5-aza-dC for 72 hours, the Slit2 mRNA expression in the treated CRC cells was significantly increased compared with the untreated control cells. Student's *t*-test; $***p < 0.001$.

Supporting Information Fig. S1*a*). The promoter methylation status of Slit2 was also investigated in CRC cell lines and 28 paired CRC/NCT tissues as well as five normal colonic epithelia samples using a MSP assay. The results showed that the methylation ratio of Slit2 in CRC tissues (21/28, 75.0%) was significantly higher than that in NCTs (13/28, 46.4%; $p = 0.029$), whereas all five normal colonic epithelia samples were unmethylated (Fig. 1*e*). Consistent with the BSP results,

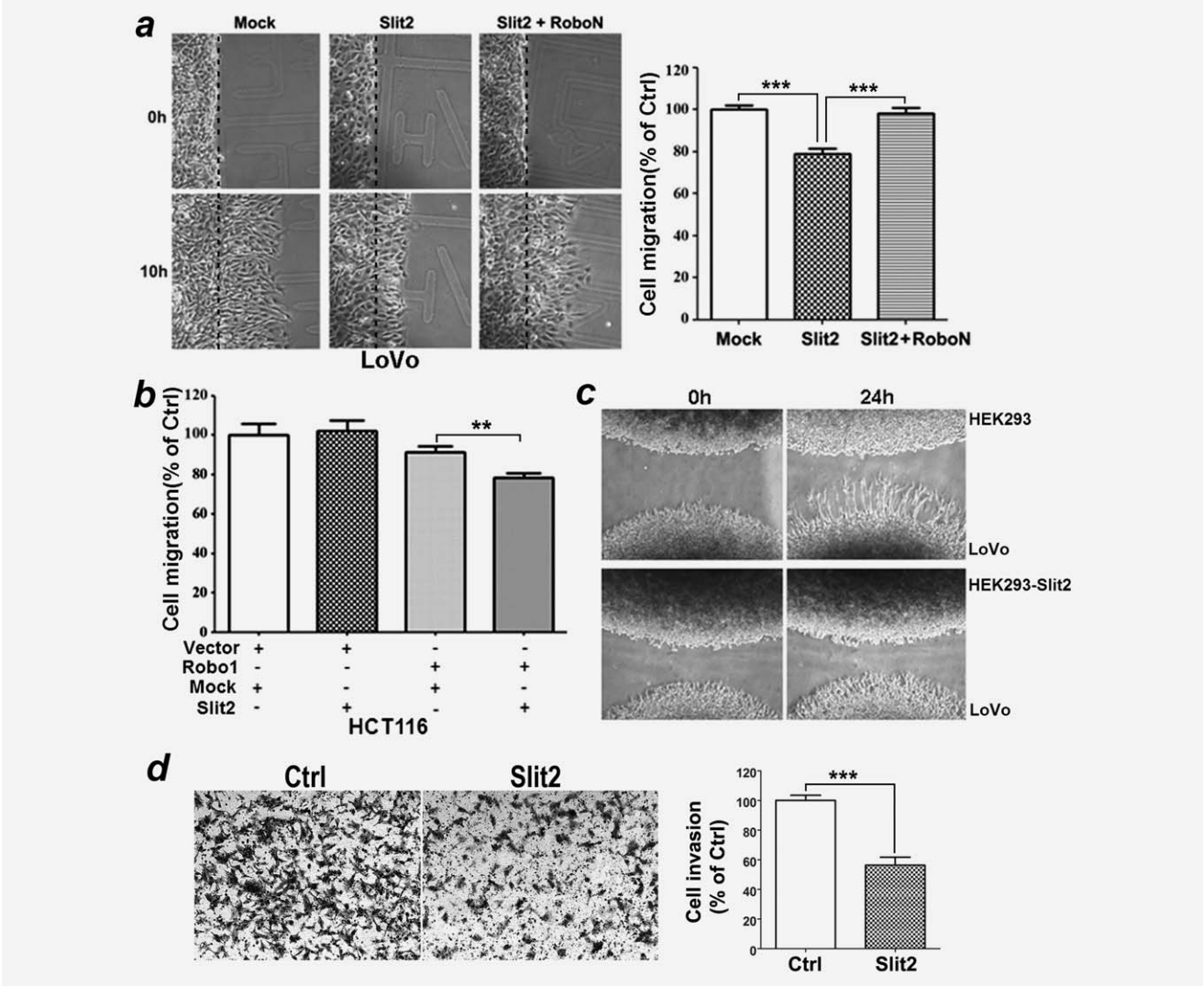

Figure 2. Slit2 inhibits CRC cell migration in a Robo-dependent manner. (*a*) Slit2 inhibits the migration of LoVo cells in the wound-healing assay. The migration of LoVo cells was examined in the presence of the mock control (Mock), Slit2 and/or RoboN conditioned medium (CM) for 10 hr after wound formation. Student's *t*-test; $***p < 0.001$. (*b*) Slit2 lacks an inhibitory effect on the migration of Robo-negative HCT116 cells. Slit2 treatment showed no significant effect on the migration of HCT116 cells lacking Robo1/2 expression, whereas transfection of Robo1 restored the inhibitory activity of Slit2 on the HCT116 cell migration. Student's *t*-test; $**p < 0.01$. (*c*) Slit2 inhibits the migration and invasion of LoVo cells in a three-dimensional coculture assay. LoVo cell aggregates embedded in collagen/Matrigel mixture were cocultured with Slit2-expressing HEK293 cell aggregates or control HEK293 cell aggregates. Images were taken 48 hr after the beginning of the coculture. (*d*) Slit2 inhibits the invasion of LoVo cells in the Transwell assay. LoVo cells transfected with Slit2 plasmid showed decreased ability to invade through Matrigel-coated membranes compared with the control. Student's *t*-test; $***p < 0.001$.

5 of the 6 CRC cell lines also showed positive results of MSP except HT29. Importantly, the CRC tumors with hypermethylated Slit2 showed decreased Slit2 expression compared with those with hypomethylated Slit2 (Supporting Information Fig. S3). To confirm that the reduced expression of Slit2 was a result of promoter hypermethylation, we treated LoVo and HCT116 cells with a demethylating agent, 5-aza-dC. Slit2 expression was restored in both cell lines treated with 5-aza-dC (Fig. 1*f*). Together, these data demonstrate that promoter hypermethylation is an important mechanism for silencing Slit2 expression in CRC.

Slit2 inhibits CRC cell migration in a Robo-dependent manner

In a significant fraction of CRC samples, Slit2 was downregulated through promoter hypermethylation, suggesting a tumor suppressive role of Slit2 in CRC. We used a modified wound-healing assay to examine the role of Slit2 in CRC cell migration.[11] LoVo cells treated with Slit2 showed decreased cell migration compared with the mock control (Fig. 2*a*). To evaluate the involvement of Robo in Slit2 signaling in CRC cells, RoboN, the soluble extracellular domain of Robo1 that blocks Slit-Robo signaling,[4,5] was used together with Slit2 in

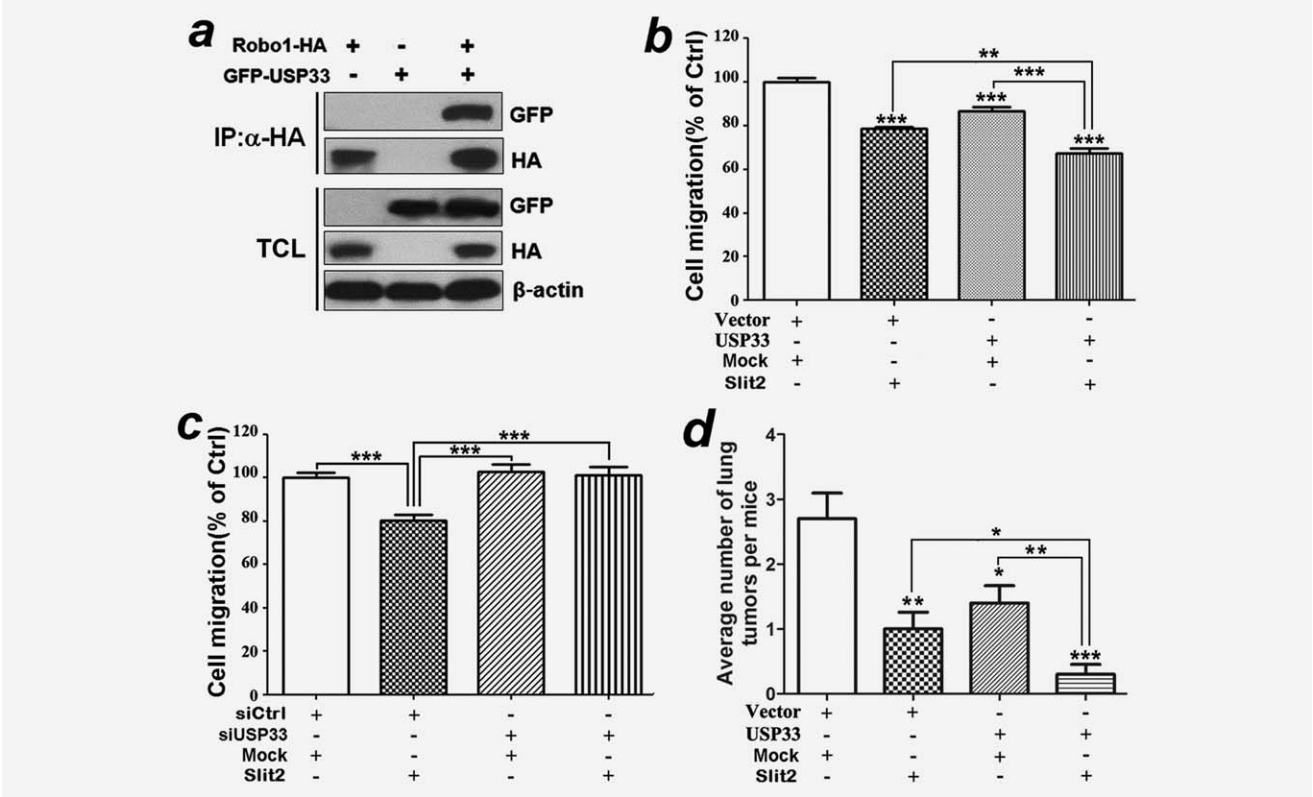

Figure 3. USP33 interacts with Robo1 and is required for mediating Slit activity in CRC cells. (*a*) USP33 interacts with Robo1. Coimmunoprecipitation was performed to detect the interaction between Robo1-HA and GFP-USP33 (*b*) in HCT116 cells transfected with Robo1-HA, or GFP-USP33. TCL panels show the expression of the corresponding proteins in the total cell lysates. Immunoprecipitates and TCL were immunoblotted with antibodies as indicated. (*b*) USP33 is required for Slit2 signaling in CRC cells. Cell migration was examined in LoVo cells transfected with GFP-USP33. Expression of USP33 enhanced the inhibitory effect of endogenous and exogenous Slit2 on the migration of LoVo cells. Student's *t*-test; $^{**}p < 0.01$; $^{***}p < 0.001$. (*c*) Downregulating USP33 blocks the inhibitory effect of Slit2 on the migration of LoVo cells. Cell migration was examined in LoVo cells transfected with siUSP33 or siCtrl. Student's *t*-test; $^{***}p < 0.001$. (*d*) USP33 enhanced the inhibitory effect of Slit2 on the *in vivo* metastasis of CRC in a lung metastasis model of nude mouse. LoVo cells stably expressing Slit2, GFP-USP33, Slit2/GFP-USP33 or the control (2×10^6) were injected into the caudal vein of each nude mouse. Student's *t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the wound-healing assay. The addition of RoboN effectively blocked the inhibitory effect of Slit2 on CRC cell migration (Fig. 2*a*). To confirm the requirement of Robo in Slit function in CRC cells, we performed cell migration analyses using HCT116 without detectable expression of Robo1 or Robo2. Treating HCT116 cells with recombinant Slit2 had no detectable effects on their migration; however, ectopic expression of Robo1 restored the inhibitory function of Slit2 on the migration of HCT116 cells (Fig. 2*b*).

To further investigate the effect of Slit2 on CRC cell migration and invasion, a three-dimensional coculture assay was performed. Migration of LoVo cells was reduced when cocultured in collagen/Matrigel matrix with HEK293 cell aggregates secreting Slit2 compared with cells cocultured with control HEK293 cell aggregates (Fig. 2*c*). In addition, we examined the effect of Slit2 in LoVo cells using a Transwell assay following transfection with Slit2-expressing plasmid or a control vector. Slit2 expression reduced cell migration and invasion in the Transwell assay (Fig. 2*d*).

We also examined the effects of Slit2 on cell proliferation. No detectable effects of Slit2 on cell proliferation were observed in any of 6 CRC cell lines (Supporting Information Fig. S4). Taken together, these data demonstrate that Slit2 inhibits CRC cell migration in a Robo-dependent mechanism.

USP33 interacts with Robo1 and mediates Slit2 signaling in CRC cells

Our previous studies have shown that USP33 interacts with Robo1 in neurons and breast cancer cells.[12,13] Western blotting analyses revealed that USP33 expression was detectable in most of the examined CRC cells, including LoVo and HCT116 cells (Supporting Information Fig. S1). To test the interaction between Robo1 and USP33 in CRC cells, we performed co-IP assays. Plasmids of Robo1-HA, GFP-USP33, or empty vector were cotransfected into HCT116 or LoVo cells. After IP using an anti-HA antibody, USP33 was detected only in the immunoprecipitates of the cells expressing both the Robo1 and USP33 proteins (Fig. 3*a*).

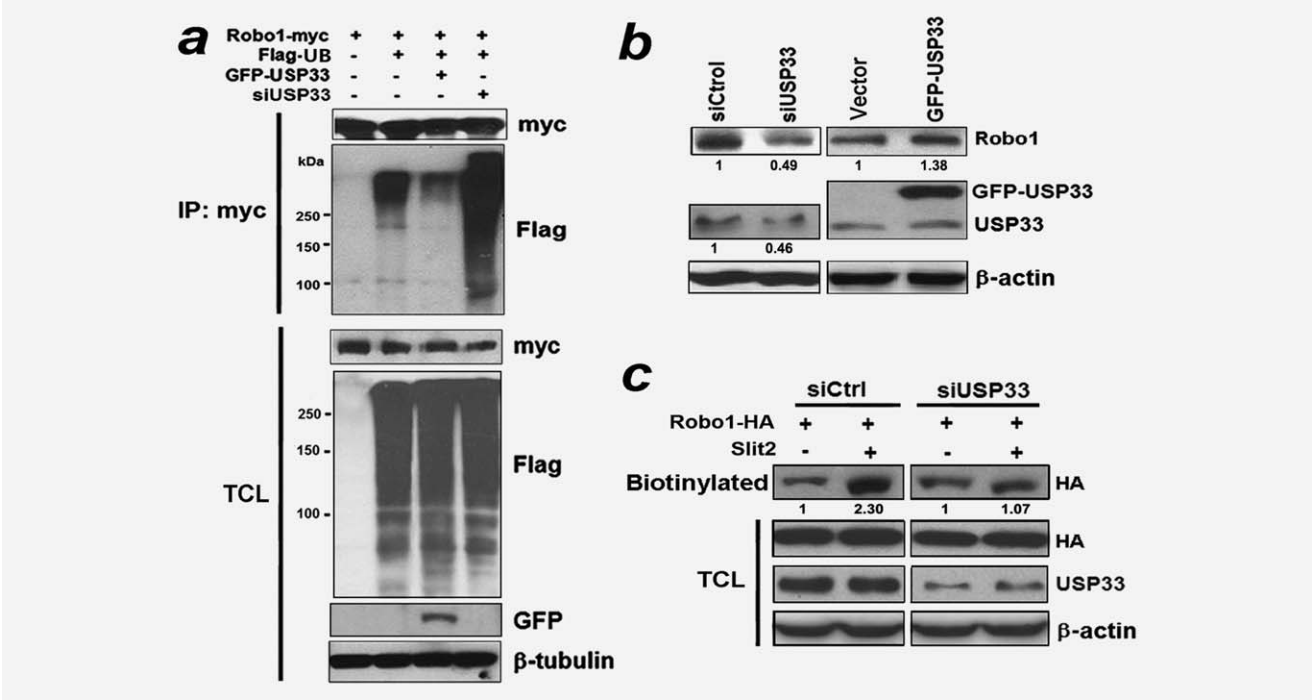

Figure 4. USP33 is involved in Silt2 signaling by deubiquitinating and stabilizing Robo1. (*a*) USP33 deubiquitinates Robo1. HCT116 cells were transfected with Robo1-myc, Flag-UB, GFP-USP33, or siUSP33 and treated with 10 μM MG132 for 6 hr. Ubiquitinylated Robo1 was detected by Western blotting using anti-Flag following immunoprecipitation of Robo1-myc with a monoclonal anti-myc antibody. TCL panels show the expression of the corresponding proteins in the total cell lysates. Expression of GFP-USP33 decreased, whereas siUSP33 increased the level of ubiquitinylated Robo1. (*b*) USP33 stabilizes Robo1. Downregulating USP33 with a specific siRNA decreased the Robo1 protein level, whereas transfection of GFP-USP33 increased the Robo1 level in LoVo cells. (*c*) Slit2 treatment increases Robo1 localization to the cell surface. Slit2 treatment significantly increased the cell surface distribution of Robo1, which was blocked by knocking-down USP33 using siUSP33, in Robo1-HA expressing HCT116 cells. Robo1-HA expressing HCT116 cells transfected with siUSP33 or siCtrl were stimulated with recombinant Slit2 for 10 min and the proteins on the cell surface were labeled by cell surface biotinylation. The biotinylated proteins were then harvested by avidin-agarose pulldown, and cell surface Robo1-HA was detected by Western blotting using anti-HA.

These results show that Robo1 interacts with USP33 in CRC cells.

To investigate the role of USP33 in mediating Slit-Robo signaling in CRC cells, we increased or inhibited USP33 expression in LoVo cells by transfecting the GFP-USP33 plasmid or siUSP33. Enhanced USP33 expression increased Slit2 inhibition of cell migration (Fig. 3*b*), whereas knocking down USP33 blocked the inhibitory effect of Slit on cell migration (Fig. 3*c*). To identify the impact of Slit2 and USP33 on *in vivo* metastasis of CRC cells, LoVo cells stably expressing Slit2 and/or USP33 were injected into the caudal vein of athymic BALB/c nude mice. Ectopic expression of Slit2 significantly reduced the number of lung metastasis sites, and USP33 overexpression enhanced the inhibitory effect of Slit2 on tumor metastasis (Fig. 3*d* and Supporting Information Fig. S5). Collectively, these data support a key role of USP33 in mediating Slit signaling in CRC cells.

USP33 deubiquitinates and stabilizes Robo1

To examine the mechanism of USP33 in regulating Slit-Robo signaling, we examined the levels of ubiquitinylated Robo1 after cotransfecting Flag-tagged ubiquitin (Flag-UB) with GFP-USP33 or siUSP33 in HCT116 cells. Following IP of Robo1-myc using a monoclonal anti-myc, ubiquitinylated Robo1 was detected using anti-Flag. Overexpression of USP33 reduced, whereas knocking-down USP33 increased the level of ubiquitinylated Robo1 (Fig. 4*a*). Western blotting analyses revealed that inhibition of USP33 expression decreased whereas overexpression of USP33 increased the Robo1 protein levels in CRC cells (Fig. 4*b*). These results show that USP33 mediates Slit-Robo signaling by deubiquitinating and stabilizing Robo1 in CRC cells (Supporting Information Fig. S6).

USP33 is required for the redistribution of Robo1 to the plasma membrane following Slit stimulation

We have reported that Slit2 stimulates the redistribution of Robo1 to the plasma membrane in an USP33-dependent manner in breast cancer and HEK cells.[11] To examine the distribution Robo1 protein in CRC cells, we performed a cell surface protein biotinylation assay in HCT116 cells following downregulation of USP33 using siRNA. As shown in Fig. 4*c*, the cell surface levels of Robo1 significantly increased 10 min after Slit2 treatment, and this Slit2-stimulated increase in cell-surface Robo1 was diminished when USP33 was knocked down by siUSP33.

USP33 expression was positively correlated with Robo1 expression in CRC tissues

To examine the potential relationship between USP33 and Robo1 expression in clinical CRC samples, we checked their protein expression in 42 CRC tumors. The protein expression of Robo1 and USP33 was observed in 58.3% (24/42) and 64.3% (27/42) of CRC tumors, respectively (Table 1 and Supporting Information Fig. S7). The protein expression of Robo1 in the CRC tissues was positively correlated with the USP33 expression (Spearman $r = 0.359$, $p = 0.020$), suggesting that USP33 acts as a regulator of Robo1 protein expression in clinical CRC tumors. The USP33 expression ratio was lower in CRC (64.3%, 27/42) than in NCT (83.3%, 35/42) ($p = 0.047$). Although the Robo1 expression ratio was lower in CRC (58.3%, 24/42) than in NCT (69.0%, 29/42), the difference was not statistical significant ($p = 0.258$).

USP33 expression is decreased in CRC samples and low USP33 expression predicts poor survival

We assessed the USP33 mRNA expression in two different CRC cohorts. We first analyzed its expression in a published human CRC microarray data set.[18] USP33 mRNA levels were reduced in CRCs compared with corresponding NCTs ($p < 0.0001$). Lower USP33 mRNA levels were associated with lymph node metastasis (Spearman $r = -0.220$, $p = 0.003$) and more advanced CRC staging (Spearman $r = -0.239$, $p = 0.001$; Fig. 5*a* and 5*b*). Kaplan-Meier analyses revealed that lower USP33 mRNA levels were associated with shorter overall survival ($p = 0.008$, Fig. 5*d*). After adjustment for age, gender, T stage, N stage, M stage and p53 mutation status, Cox multivariate analyses showed that the USP33 mRNA level was an independent prognostic factor for CRC survival. Tumors with higher USP33 expression levels presented lower death risk (HR = 0.491, 95% CI = 0.278–0.870, $p = 0.015$). To further assess the value of USP33 in CRC prognosis, we examined its mRNA expression in samples from an independent cohort of 79 CRCs (Figs. 5*c* and 5*e*). Approximately 50% of these CRC tumors showed decreased USP33 levels compared with their corresponding NCTs ($p < 0.0001$, Fig. 5*c*). Higher USP33 expression was correlated with better survival probability ($p = 0.028$, Fig. 5*e*). These data support USP33 as a new prognostic factor for CRC.

Table 1. The protein expression of Robo1 and USP33 in human CRC tissues

	USP33 expression		
	Negative	Positive	Total
Robo1 expression			
Negative	10	8	18
Positive	5	19	24
Total	15	27	42

Chi-square = 5.401, $p = 0.020$.

Discussion

Slit-Robo signaling plays an important role in regulating the critical process of cell migration in a variety of tissue types in addition to its role in neuronal guidance in the nervous system.[6,20] In this study, we demonstrated that Slit2 is downregulated in CRC samples and inhibits CRC cell migration in a Robo-dependent manner. In CRC cells, USP33 interacts with Robo1 and mediates the inhibitory activity of Slit2 on CRC cell migration by deubiquitinating and stabilizing Robo1. In addition, USP33 is required for the increased redistribution of Robo1 to the plasma membrane in response to Slit2 treatment. USP33 expression is reduced in CRC samples. Lower USP33 expression correlates with poorer prognosis in CRC patients. These data support the tumor suppressive roles of Slit2 and USP33 in CRC.

Previous studies have shown that Slit2 expression was downregulated in human cancers, including CRC.[8] The first evidence that Slit-Robo signaling may be involved in cancer came from a study by Sundaresan *et al.*[21] They reported homozygous deletions at the 3p12 locus, which is the chromosomal region in which the human Robo1 gene resides, in breast and lung cancer. Further studies have revealed that a loss of heterozygosity (LOH) at the Slit2 gene locus (4q25–26) was frequently found in breast carcinoma, cervical carcinoma, lung carcinoma, mesothelioma and cervical cancer.[22–24] Our analyses show that approximately 4%–7% of CRC samples carried Slit2 mutations (Supporting Information Fig. S2). Furthermore, a common mechanism for silencing Slit2 expression in human cancers appears to be promoter hypermethylation. Epigentic inactivation of the Slit2 and Slit3 genes has been reported in a wide range of human cancers.[7,8,19,20,25,26] Consistent with previous reports,[8,27] our data show that extensive promoter hypermethylation of Slit2 is detected in CRC tissues, suggesting that promoter hypermethylation is an important mechanism for Slit2 silencing in CRC. Interestingly, the Slit2 methylation status was proposed to be a new CRC biomarker in a recent work.[28] Although overwhelming evidence has shown Slit down regulation in different types of cancers,[7,8,19,20,25,26,28,29] a few reported that the Slit genes were upregulated in some types of human cancers.[30,31] The controversial results, even in the same type of tumor,[29,30] may be due to the small case numbers in these studies or different tumor types. Further studies are necessary to resolve this controversy.

Slit2 regulates cell migration in neuronal and non-neuronal cells.[6] No significant effect of Slit2 on cell proliferation was detected in CRC cells. Our data demonstrated that Robo-positive CRC cells treated with recombinant Slit2 showed attenuated cell migration; and Slit2 lost its inhibitory activity on cell migration in Robo-negative CRC cells. In addition, Slit2 inhibition of cell migration was reversed by treatment with soluble Robo extracellular domain. These results demonstrate that Slit2 inhibits CRC cell migration in a Robo-dependent manner.

A number of Robo-interacting proteins, including Abl,[32] Dock,[33] ERK1/2,[34] srGAPs[13] and Vilse,[35] have been identified

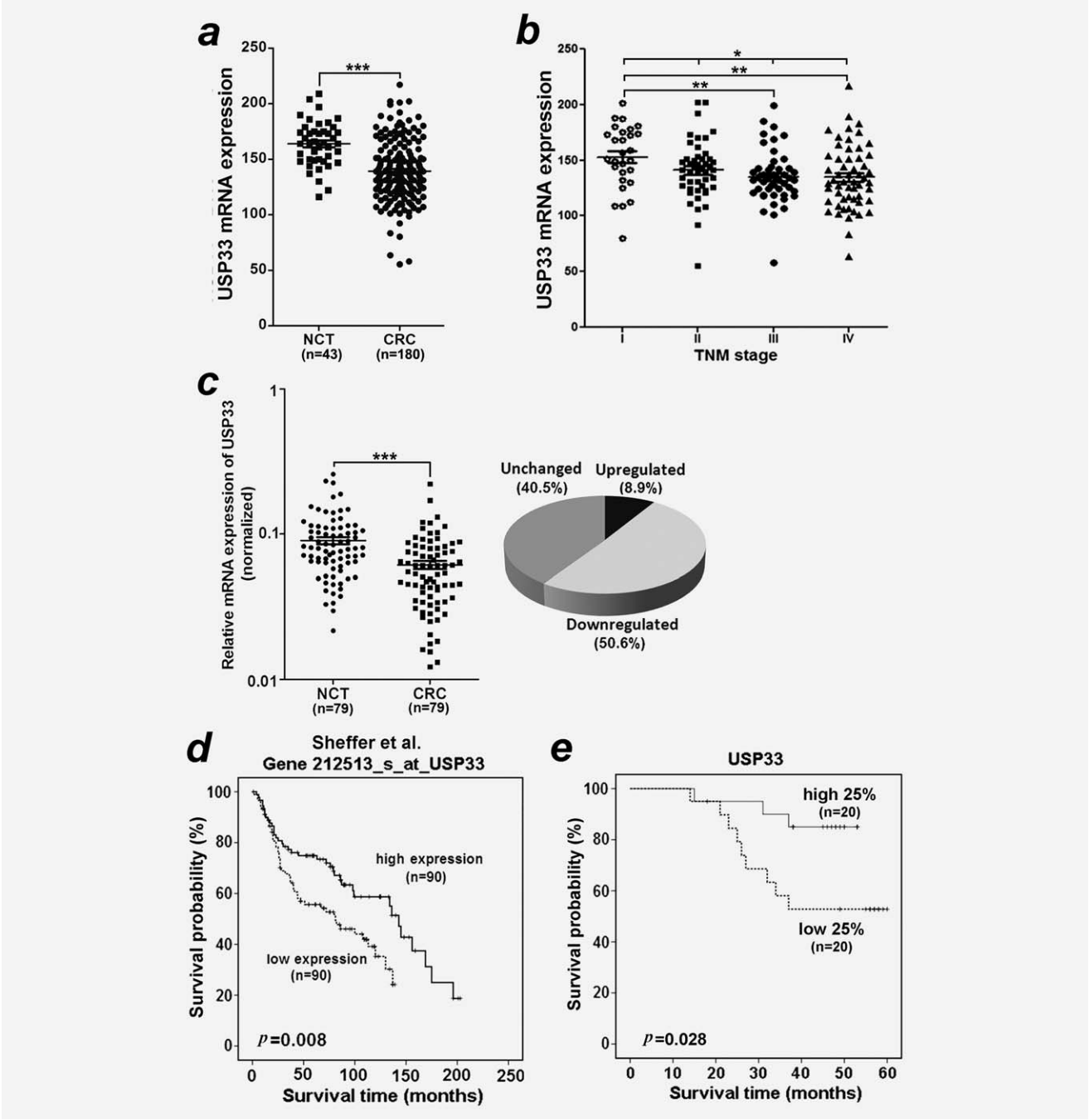

Figure 5. USP33 expression is downregulated in CRC and reduced USP33 expression predicts poor survival. (*a*) USP33 expression is reduced in CRC samples from a published CRC microarray dataset. Mann–Whitney *U* test; ***$p < 0.001$. (*b*) USP33 mRNA expression is negatively correlated with tumor TNM stage. Mann–Whitney *U* and Kruskall–Wallis tests; *$p < 0.05$; **$p < 0.01$. (*c*) USP33 expression is decreased in CRC tissues from an independent cohort. The mRNA expression of USP33 in 79 paired CRC and NCTs was quantitated using real time PCR. About half of the tumors (50.6%, 40/79) showed decreased USP33 mRNA levels (more than 1.5-fold) compared with their corresponding NCTs. Mann–Whitney *U* test; ***$p < 0.001$. (*d* and *e*) Lower mRNA levels of USP33 are associated with shorter overall patient survival. Survival analyses were performed using the Kaplan-Meier method based on the USP33 mRNA expression from Sheffer's and test cohorts. Cases were divided into two (*d*) or four groups (*e*) according to the USP33 mRNA levels. The expression profile data (*a*, *b* and *d*) was obtained from the paper of Sheffer *et al.*[18]

that may mediate Slit-Robo signaling in different systems. USP33, a recently identified Robo1-interacting molecule, is required for Slit-Robo signaling in regulating the midline crossing of commissural neuron axons[12] and in suppressing breast cancer cell migration.[11] USP33 was originally identified as a downstream target for ubiquitination and degradation by pVHL E3 ligase.[14] Several proteins, including DIO2,[36] ADRB2,[37] hSP56,[38] beta-arrestin,[39] RALB[40] and CP110,[41]

have been identified as USP33-interacting partners, indicating extensive biological functions of USP33 in human physiological and pathological processes. In this study, we demonstrated that USP33 is downrgulated in CRC samples and is required for Slit2 signaling in suppressing CRC cell migration. USP33 mediates Slit2 signaling by deubiquitinating and stabilizing Robo1. In addition, USP33 is required for the redistribution of Robo1 to the cell surface in response to Slit2 treatment, which was previously reported in breast cancer cells.[11] The data presented in this study demonstrate that USP33 utilizes two different mechanisms to mediate Slit signaling in CRC cells, stabilizing the Robo1 protein and regulating the cell surface localization of Robo1. Based on these results, we propose a model for the Slit-Robo-USP33 signal transduction pathway in CRC cells (Supporting Information Fig. S4). In our previous study, we showed that USP33 was required for the Slit2-stimulated redistribution of Robo1 to the cell surface in breast cancer cells. However, USP33 did not affect Robo1 protein levels in breast cancer cells.[11] These data suggest that different types of cancer cells may use different combinations of molecular pathways.

Recent reports proposed other molecular mechanisms by which Slit-Robo signaling regulate CRC tumorigenesis. Two groups reported different results on the impact of Slit-Robo signaling in CRC cells. Zhou *et al.*[9] showed that treatment with the amino-terminal domain of Slit2 induced malignant transformation of colorectal epithelial cells through Hakai-mediated E-cad ubiquitination and lysosomal degradation during colorectal epithelial cell carcinogenesis, and proposed that Slit-Robo signaling may induce the epithelial-mesenchymal transition (EMT), tumor growth and liver metastasis. On the contrary, Chen *et al.*[10] reported that Slit2 inhibited CRC cell migration and EMT by negatively regulating AKT-GSK3β signaling. A similar function of Slit2 in inhibition of cell migration via β-catenin has been reported in breast cancer cells.[42] It is critical to have more systematic studies to elucidate the role of Slit-Robo signaling in CRC.

Our detailed analyses in different CRC cohorts confirmed the downregulation of USP33 expression in CRC samples and revealed that USP33 mRNA levels were negatively correlated with lymph node metastasis and tumor TNM stages. Higher USP33 expression is associated with longer patient survival, and further analyses suggest that the downregulation of USP33 in CRC could be partially attributed to heterozygous deletion at the USP33 gene locus (data not shown). The USP33 mRNA expression level has potential value in serving as an independent prognostic factor for CRC. In addition, the protein expression of USP33 and Robo1 was also examined in a small CRC cohort, and the results showed that USP33 expression was positively correlated with Robo1 expression, indicating the regulating role of USP33 on Robo1 in clinical CRC tumors. Due to the small case number and weak staining strength of Robo1, we did not observed significant downregulation of Robo1 in CRC and significant effect of Robo1 on survival (data not shown). Zhou *et al.*[9] examined the Robo1 protein using an anti-Robo1 antibody produced by their lab and reported that high Robo1 protein expression in CRC associated with tumor metastasis and poor survival. So the exact effect of Robo1 on survival should be independently validated using commercial anti-Robo1 antibodies with good specificity and sensitivity in large CRC cohorts in future work.

In summary, our data reveals USP33 as a new player in CRC and Slit-Robo-USP33 as a previous unknown signal transduction pathway in suppressing CRC cell migration. Ubiquitinating and deubiquitinating enzymes have been the subjects of intense studies in cancers. Modulators of the enzymes are being evaluated as potential agents in cancer therapy. Future studies are needed to assess the potential value of genes in the Slit-Robo-USP33 pathway in the diagnosis and treatment of CRC.

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